ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN TWO-DIMENSIONAL ELECTROPHORESIS

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Introduction

Most one-dimensional (1-D) methods of polyacrylamide gel electrophoresis are limited to the resolution of 100 or so protein zones. These techniques are therefore not suitable for the analysis of complex mixtures containing several thousands of proteins, such as total protein homogenates of whole cells and tissue. In addition, they are only able to separate proteins on the basis of a single physico-chemical property. For example, the observation of a particular zone following SDS-PAGE does not imply protein heterogeneity, but simply indicates that any proteins present in that zone have nearly identical size properties, while their charge (and other) properties could be very different.

The best approach to this problem is to combine two different 1-D methods into a 2-D procedure. Ideally, the methods used for each dimension should be selected by their ability to separate proteins according to different properties in each dimension. Thus, if each method when used alone is able to resolve 100 protein zones, it would be expected that up to 10 000 proteins might be resolved when these methods are used orthogonally. This level of resolution has rarely been achieved in practice, but nevertheless 2-D has become the method of choice for the analysis of patterns of protein expression in whole cells, tissues and organisms; the area now known as proteomics.

History of 2-D

The first protein separation by 2-D is attributed to Smithies and Poulik who in 1956 described a combination of paper and starch gel electrophoresis for the separation of serum proteins. Since that time, subsequent advances in electrophoresis, such as the use of polyacrylamide gels, discontinuous buffer systems, gradient gels, SDS-PAGE, and isoelectric focusing (IEF) have all resulted in the development of improved methods of 2-D. These developments culminated in the 1970s with publications from several independent groups describing a combination of a first-dimension separation by IEF under denaturing conditions with a second dimension separation by SDS-PAGE. This coupling of IEF with SDS-PAGE resulted in a method of 2-D which separates proteins according to two independent parameters, charge and size.

The O'Farrell Method of 2-D

The method described by O'Farrell in 1975 has formed the basis of almost all subsequent developments in 2-D, and several thousand papers have been published using this technique in the 25 years following its publication. This method was optimized for the separation of the proteins of *Escherichia coli* (*E. coli*) and used a combination of IEF in cylindrical gels (cast in glass capillary tubes) containing 8 M urea and 2% w/v of the non-ionic detergent, Nonidet P-40 (NP-40), with the SDS-PAGE system of Laemmli. This method was able to resolve around 500 proteins from *E. coli*. It has subsequently been applied to a wide variety of samples.

Limitations of the O'Farrell Method

The main problem with the 2-D method of O'Farrell is associated with the synthetic carrier ampholytes (SCA) which are used to generate the pH gradient in the IEF dimension. SCA are produced by a complex synthetic process which is difficult to control reproducibly. This results in considerable batch-to-batch variability and limits the reproducibility and consistency of 2-D separations. Perhaps more importantly SCA are relatively small molecules, which are not fixed within the IEF gel. As a consequence, the electroendosmotic flow of water that occurs during IEF results in migration of the SCA molecules towards the cathode.

This process, known as 'cathodic drift', results in pH gradient instability and is exacerbated using tube gels due to the negatively charged groups present on the walls of the glass capillaries. In practice, pH gradients using the O'Farrell method of 2-D rarely extend far beyond pH 7, with the resultant loss of the basic proteins. This problem was recognized by O'Farrell, who developed an alternative procedure,

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known as non-equilibrium pH gradient electrophoresis (NEPHGE), for the 2-D separation of basic proteins. In this method, separation occurs on the basis of protein mobility in the presence of a rapidly forming pH gradient, but reproducibility is extremely difficult to control. Fortunately, this problem was solved with the development of immobilized pH gradient (IPG) IEF.

2-D Using IPG IEF

IPG IEF gels are prepared using Immobilines (Amersham Pharmacia Biotech), a series of eight acrylamide derivatives with the structure $CH_2=CH–CO–NH–R$, where R contains either a carboxyl or tertiary amino group. These form a series of buffers with different pK values distributed through-

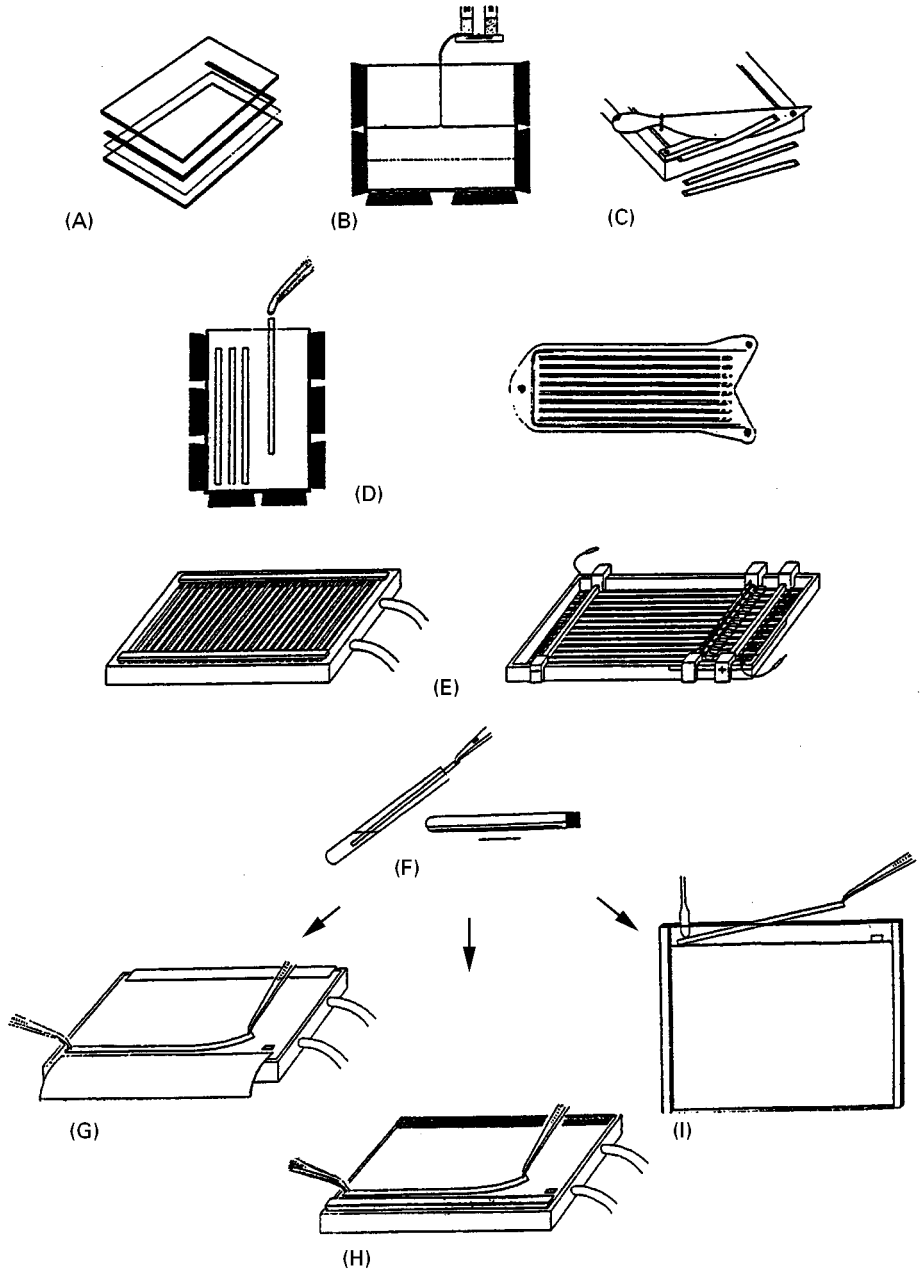

Figure 1 Schematic diagram of the procedure of 2-D using IPG IEF. (A) Assembly of the polymerization cassette for the preparation of IPG and SDS gels cast on plastic backings, (B) casting of IPG and gradient SDS gels, (C) cutting of washed and dried IPG gels into individual IPG strips, (D) rehydration of IPG strips, (E) IEF in individual IPG strips, (F) equilibration of IPG strips prior to SDS-PAGE, (G) transfer of IPG strip onto surface of laboratory-made horizontal SDS gel along cathodic wick, (H) transfer of IPG strip onto surface of commercial horizontal SDS gel along cathodic buffer strip, (I) loading of IPG strip onto the surface of a vertical SDS gel. (Courtesy of A. Görg, Technical University, Munich, Germany).

out the pH range 3 to 10. The appropriate IPG reagents, calculated according to published recipes, are added to the mixture used for gel polymerization. Thus, during polymerization, the buffering groups which will form the pH gradient are covalently attached via vinyl bonds to the polyacrylamide backbone. IPG generated in this way are, therefore, immune to the effects of electroendosmosis, so that they provide the opportunity to carry out IEF separations which are extremely stable, allowing the true equilibrium state to be attained.

Initial attempts to implement the IPG technology to 2-D separations encountered several problems. Fortunately, largely due to the work of Görg and her colleagues, these problems have been solved and IPG IEF has become the method of choice for the first dimension separation of 2-D. The method is shown schematically in **Figure 1**. Briefly, IPG slab gels of the desired pH range are cast (**Figure 1**(B)) according to the extensive library of published recipes. After polymerization, the gels are washed, dried and stored at − 20°C. The required number of gel strips (3–5 mm wide) for 2-D are cut off of the slab using a paper cutter (**Figure 1**(C)). Alternatively, a range of ready-made strips is available commercially from Amersham Pharmacia Biotech. IPG strips of any desired length can be used, but is should be remembered that, in general, the larger the separation area of a 2-D gel, the more proteins can be resolved. Strips of 18 cm are usually employed for high-resolution separations, while shorter strips (7 or 11 cm) are used for rapid screening applications.

A choice of a linear pH gradient from 3.5 to 10 is often useful for the initial analysis of a new type of sample. However, for many samples this can result in loss of resolution in the region of pH 4 to 7, in which the pI values of many proteins occur. This problem can be overcome to some extent with the use of a non-linear pH 3.5–pH 10 IPG IEF gel, in which the pH 4–7 region contains a much flatter gradient than in the pH 7–10.5 region. This allows good separation in the pH 4–7 region while still resolving the majority of the more basic species (**Figure 2**). However, use of a pH 4–7 IPG IEF gel will result in even better protein separation (**Figure 3**). Commercial IPG strips are available for these pH ranges (Amersham Pharmacia Biotech). Laboratory-made IPG strips with either very narrow pH gradients (spanning 1 pH or less) can be useful for separating components with very similar pI values, while very basic pH gradients can be used to advantage for certain types of sample, such as ribosomal proteins and nuclear proteins.

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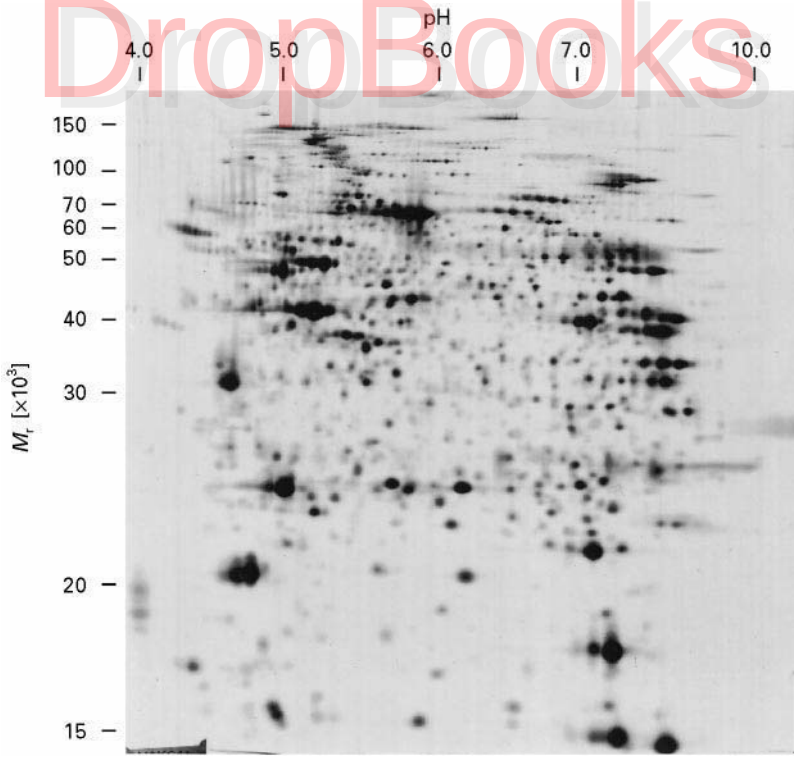

Figure 2 A 2-D separation of 100 μg heart proteins using a nonlinear pH 3.5 to 10 IPG IEF gel in the first dimension. The protein pattern was visualized by silver staining. The scale at the top indicates the nonlinear pH gradient obtained using an IPG 3-10 NL strip for the first dimension IEF separation. The scale at the left indicates the size separation in the range 15 to 150 kDa using a 15% SDS-PAGE gel in the second dimension.

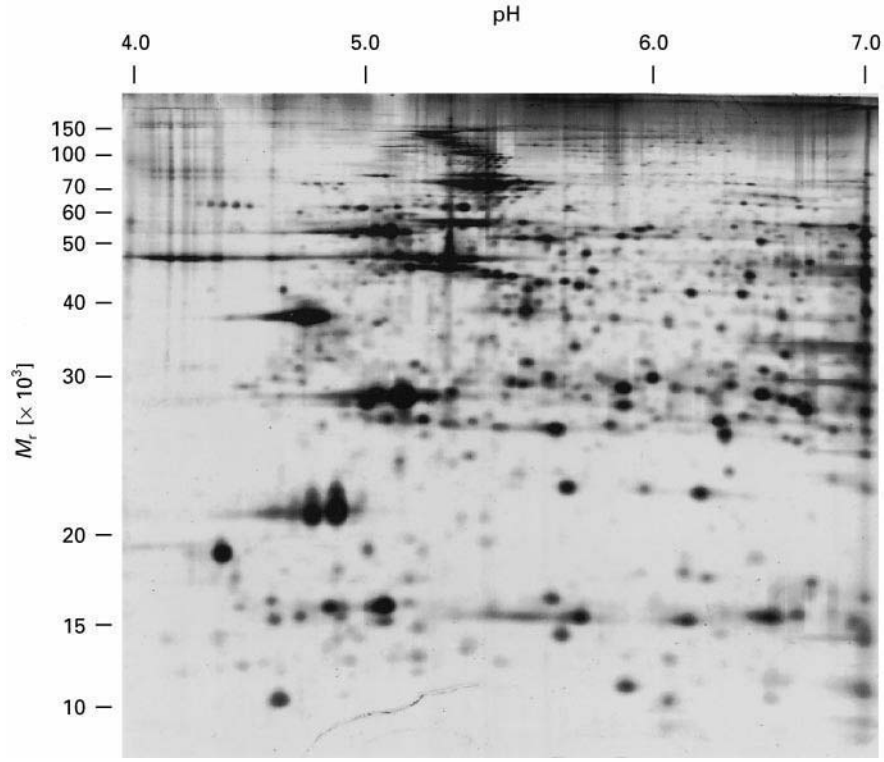

Figure 3 A 2-D separation of 100 μg heart proteins using a linear pH 4 to 7 IPG IEF gel in the first dimension. The protein pattern was visualized by silver staining. The scale at the top indicates the linear pH gradient obtained using an IPG 4-7 strip for the first dimension IEF separation. The scale at the left indicates the size separation in the range 10 to 150 kDa using a 10% SDS-PAGE gel in the second dimension.

For use in 2-D, the strips are rehydrated in a re-swelling cassette (**Figure 1**(D)) in a solution containing 8 M urea, 0.5% non-ionic (e.g. NP-40, Triton X-100) or zwitterionic (CHAPS) detergent (3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate), 15 mM DTT and 0.2% synthetic carrier ampholyte (SCA) of the appropriate pH range. The strips are then placed directly on the surface of the cooling plate of a horizontal flat-bed electrophoresis apparatus (**Figure 1**(E)). A convenient alternative is to use the special strip tray available from Pharmacia (**Figure 1**(E)). This tray is fitted with a corrugated plastic plate which contains grooves allowing easy alignment of the IPG strips. In addition, the tray is fitted with bars carrying the electrodes and a bar fitted with sample cups allowing the application of samples at any desired point on the gel surface. This tray is filled with silicone oil which protects the gel from the effects of the atmosphere during IEF. Horizontal streaking can often be observed at the basic end of 2-D protein profiles, particularly when IPG 6–10 is used for the first dimension. This problem can be resolved by applying an extra electrode strip soaked in 15 mM DTT on the surface of the IPG strip alongside the cathodic electrode strip. This has the advantage that the DTT within the gel, which migrates towards the anode during IEF, is replenished by the DTT released from the strip at the anode. An alternative approach is to use the non-charged reducing agent, tributyl phosphine (TBP), which does not migrate during IEF and has been found to greatly improve protein solubility during IEF.

Sample Preparation

There is no universal method of sample preparation for 2-D due to the diverse nature of samples which can be analysed. Whatever method is used, it is essential to minimize protein modifications which can result in artefactual spots on 2-D protein patterns. In particular, samples containing urea should not be heated as this will lead to charge heterogeneity as a result of protein carbamylation by isocyanate ions formed from the decomposition of urea. Proteases present within samples can also readily result in artefactual spots, so that samples should be subjected to minimal handling and kept cold at all times. Protease inhibitors can also be added.

Liquid samples containing a relatively high protein concentration (e.g. serum, plasma) require little or no

pre-treatment prior to 2-D. However, less concentrated solutions (e.g. urine, cerebrospinal fluid (CSF), amniotic fluid) often require concentration by methods such as lyophilization, or precipitation with trichloroacacetic acid (TCA) or acetone. Solid tissue samples must usually be disrupted in the presence of solubilization solution. For small samples this is readily achieved by crushing the sample in liquid nitrogen using a pestle and mortar, while larger tissue samples must be homogenized using a suitable device. Cell suspensions can be readily harvested by centrifugation, while cells adherent to a substrate, such as a tissue culture flask or dish, should be collected by scraping (the use of proteases should be avoided to prevent possible sample degradation). Alternatively, the cells can be detached by lysis directly in a small volume of sample solubilization solution.

Sample Solubilization

The most popular method for protein solubilization for 2-D is that originally described by O'Farrell, using a mixture of 9.5 M urea, 4% w/v NP-40, 1% w/v DTT and 2% w/v SCA. While this method works well for the majority of samples, it is not universally applicable, with membrane proteins representing a particular challenge. The zwitterionic detergent, CHAPS has been found to be effective for the solubilization of membrane proteins, particularly when used at a concentration of 4% w/v in combination with a mixture of 2 M thiourea and 8 M urea. Linear sulfobetaine detergents, such as SB 3–10 or 3–12, are also effective solubilizing agents, but these are not compatible with high concentrations of urea. This can be overcome by using these reagents at 2% w/v in combination with 5 M urea, 2 M thiourea and 2% CHAPS.

The presence of nucleic acids can be problematic during IEF. This is due to an increase in the viscosity of the sample and in some cases formation of complexes with the sample proteins, leading to artefactual migration and streaking. If problems of this type are suspected, it is best to degrade the nucleic acid by the addition of a suitable pure (i.e. protease free) endonuclease to the sample solubilization solution.

Sample Reduction

Protein disulfide bonds are normally reduced with free thiol-containing reagents such as DTT or β-mercaptoethanol. However, reagents such as DTT are charged so that they migrate out of the gel during IEF, leading to reoxidation of the sample proteins which can result in loss of sample solubility. It has recently been reported that replacing the thiol-containing reducing agents with a non-charged reducing agent such as tributyl phosphine (TBP) can greatly increase protein solubility during the IEF dimension and result in increased transfer to the second dimension gel.

Sample Application and Running Conditions

Samples are usually applied into silicone rubber frames or special sample cups (**Figure 1**(E)) placed either at the anodic or cathodic end of the IPG strips, the optimum position being determined empirically for each type of sample. The initial voltage should be limited to 150 V for 30 min to allow maximal sample entry and then progressively increased until 3500 V is attained. The time required for the run depends on several factors, including the type of sample, the amount of protein applied, the length of the IPG strips, and the pH gradient. The IEF run should be performed at 20°C, as at lower temperatures there is a risk of urea crystallization and higher temperatures have been found to result in alterations in the relative positions of some proteins on the final 2-D patterns. Some typical running conditions are given in **Table 1**.

This method of sample application can result in protein precipitation and the effect is more pronounced when high protein loadings (1 mg or more) are used. The problem can be overcome by reswelling the IPG strips directly in the solution containing the protein sample to be analysed. Very high protein loads (>10 mg) have been successfully separated using this method, but there can be a selective loss of high molecular weight, very basic and membrane proteins. Recently a new integrated instrument, named the IPGPhor (Amersham Pharmacia Biotech), has been developed to simplify the IPG IEF dimension 2-D. This instrument features a strip holder that provides for the rehydration of individual IPG strips with or without sample, optional separate sample loading, and subsequent IEF, all without handling the strip after it is placed in the ceramic strip holder. The

Table 1 Suggested running conditions for 18 cm IPG strips for the first, IEF dimension of 2-D. The strips should be run at 0.05 mA per strip (2 mA maximum total), 0.5 W maximum, 20°C

Voltage (maximum)	*IPG strip (pH range)*	*Time*
150	All	30 min
300	All	60 min
1500	All	60 min
3500	4–7	42 000 Vh
	4–8	35 000 Vh
	4–9	30 000 Vh
	6–10	35 000 Vh
	3–10.5	25 000 Vh

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instrument can accommodate up to 12 individual strip holders and incorporates Peltier solid-state cooling and a programmable 8000 V, 1.5 mA power supply.

Equilibration Between Dimensions

After the IPG IEF dimension, strips can be used immediately for the second dimension. Alternatively, strips can be stored between two sheets of plastic film at $-80^{\circ}C$ for periods of several months. Prior to the second-dimension separation, it is essential that the IEF gels are equilibrated to allow the separated proteins to interact fully with sodium dodecyl sulfate (SDS) so that they will migrate properly during SDS-PAGE (Figure 1(F)). The recommended protocol is to incubate the IPG IEF gel strips for 15 min in 50 mM Tris buffer, pH 8.8 containing 2% w/v SDS, 1% w/v DTT, 6 M urea and 30% w/v glycerol. The urea and glycerol are used to reduce electroendosmotic effects which otherwise result in reduced protein transfer from the first to the second dimension. This is followed by a further 15 min equilibration in the same solution containing 5% w/v iodoacetamide in place of DTT. The latter step is used to alkylate any free DTT, as otherwise this migrates through the second-dimension SDS-PAGE gel, resulting in an artefact known as 'point-streaking' which can be observed after silver staining. An alternative procedure, allowing equilibration to be achieved in a single step, is to replace the DTT in the equilibration buffer with 5 mM TBP, which is uncharged and so does not migrate during SDS-PAGE.

The Second Dimension

After equilibration, the first dimension IEF gels are applied directly to the surface of the second-dimension SDS-PAGE gels. The SDS-PAGE gels can be of any appropriate single or gradient polyacrylamide, and can be used either in a vertical (**Figure 1**(I)) or horizontal format (**Figure 1**(G), 1(H)). The use of vertical formats enables multiple gels to be run simultaneously, which improves reproducibility, while the use of horizontal, 0.5 mm thin SDS gels cast on plastic supports improves the ease of handling the gels and gives rapid separations.

Resolution of 2-D

The resolving capacity of 2-D gels is usually considered to be proportional to the total gel area available for the separation. Using 18 cm long IPG IEF gels in combination with 20 cm long second-dimension SDS-PAGE gels, around 2000 proteins can be readily resolved. Only a few hundred proteins can be separated using mini-gel formats, but these are much quicker to run and can be useful for rapid screening purposes. For maximum resolution of very complex mixtures, very large format gels (>30 cm in each dimension) can be used. These are reported to be able to separate as many as 5000 to 10 000 proteins from whole cell lysates, but this is achieved at the expense of the ease of gel handling and processing.

Reproducibility of 2-D

Until recently reproducibility was a major problem limiting the more widespread application of 2-D. Using the tube gel technique of O'Farrell, it was often difficult to obtain reproducible separations of a particular type of sample even within a single laboratory, while comparison of 2-D separation patterns generated in different laboratories was often considered to be impossible. The use of dedicated equipment for 2-D, such as the ISO-DALT (Amersham Pharmacia Biotech) and the Investigator (ESA Inc) systems, helps in this regard as it allows the simultaneous electrophoresis of large numbers (between 5 and 20) of 2-D gels under reproducibly controlled conditions. More importantly, inter-laboratory studies of various types of sample (heart, barley, yeast) have unequivocally demonstrated that 2-D using IPG IEF results in 2-D protein separations with very high spatial and quantitative reproducibility.

Proteomics

2-D separation has now matured into a technique which is capable of separating reproducibly thousands of proteins present in samples such as cells, tissues and even whole organisms. Recent developments in methods for the microchemical characterization of proteins, particularly techniques for the analysis of proteins and peptides by mass spectrometry, now make it possible to identify and characterize proteins spots directly from 2-D gels. This has made 2-D separation an ideal tool to use in studies designed to determine the nature and function of the large number of structural genes being identified in various genome initiatives. This area has become known as 'proteomics' and is the subject of a separate article.

Further Reading

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3. ABBREVIATIONS

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2,3,4,6-TeCP	2,3,4,6-tetrachlorophenol
2,3-DMP	2,3-dimethylpentane
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4,5-TP	2-(2,4,5-trichlorophenoxy)propionic acid
2,4,6-TCP	2,4,6-trichlorophenol
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
2,4-DMP	2,4-dimethylphenol
2-CP	2-chlorophenol
2-DNP	2-dinitrophenol
2-M-4,6-DNP	2-methyl-4,6-dinitrophenol
2-NP	2-nitrophenol
4-NP	4-nitrophenol
AA	amino acid
AAA	amino acid compositional analysis
AAS	atomic absorption spectrometry
ACN	acetonitrile
ADAM	9-anthryldiazomethane
ADC	analog-to-digital converter
AD-CSP	amylose *tris*-(3,5-dimethylphenylcarbamate) CSP
AE	alcohol ethoxylates
AEDA	aroma extract dilution analysis
AFM	atomic force microscopy
AGP	α_1-acid glycoprotein